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Selenium Chemoprevention of Prostate Cancer

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| 14. ABSTRACT The present study is to investigate the functional significance and mechanism of selenium suppression of hTERT in human prostate cancer. We found that over-expression of hTERT attenuates the apoptosis inducing activities of selenium, supporting an important role of hTERT in selenium action in prostate cancer cells. More importantly, we found that combined bicalutamide (an anti-androgen) and selenium treatment further decreases AR transcriptional activity, hTERT expression and induces greater apoptosis than single treatment alone, indicating that selenium in combination with anti-androgen could represent a viable approach to improve the therapeutic outcome of androgen deprivation therapy. We also found that selenium can induce DNA damage response in LNCaP cells. In addition, our data showed that androgen-stimulated AR signaling induces the expression of hTERT through up-regulating hTERT promoter activity. Selenium can block the induction of hTERT by androgen, suggesting that AR signaling is mediating the inhibitory effect of selenium on hTERT expression. However, over-expression of AR is not able to reverse the effect of selenium on hTERT expression. The data indicate that in addition to change of AR protein level, other mechanism(s) might involve in this process. Additionally, we found that prostate cancer cells expressing wild-type or mutant AR respond differentially to androgen in term of hTERT expression. | | | | | |
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A. INTRODUCTION:

A major goal of this proposal is to investigate telomerase as a potential target of androgen receptor (AR) signaling suppression by selenium. The reasons for focusing on selenium-AR-telomerase axis are as follows. (a) Telomerase activation has been reported in >90% of prostate cancer samples and in all human prostate cancer cell lines, but not in normal or benign prostatic hyperplasia tissues (1-5). The inhibition of telomerase by molecular intervention has been shown to limit life span, impair cell growth, and suppress the tumorigenic potential of cancer cells of different organs (including prostate), thus making it an attractive target for prostate cancer prevention and treatment (6-9). (b) AR signaling has been reported to regulate telomerase activity and telomere reverse transcriptase (hTERT) expression (10-12), suggesting that suppression of AR signaling is a viable approach for blocking telomerase activation. (c) Our previous reports showed that selenium reduces the abundance of AR protein, thus leading to the suppression of AR trans-activation and the down-regulation of AR-target genes (13;14). Considering the role of AR signaling in regulating telomerase activity and the importance of telomerase activation in prostate carcinogenesis, it is imperative to study whether AR signaling suppression by selenium may contribute to a reduction of hTERT expression and telomerase activity. The findings from this proposal will provide a justification for a mechanism-driven strategy in using selenium to control prostate cancer development and progression. During this first year of funding period, we examined the biological significance of hTERT/telomerase suppression in mediating the anti-cancer effect of selenium and the mechanistic basis for hTERT/telomerase regulation by AR signaling.

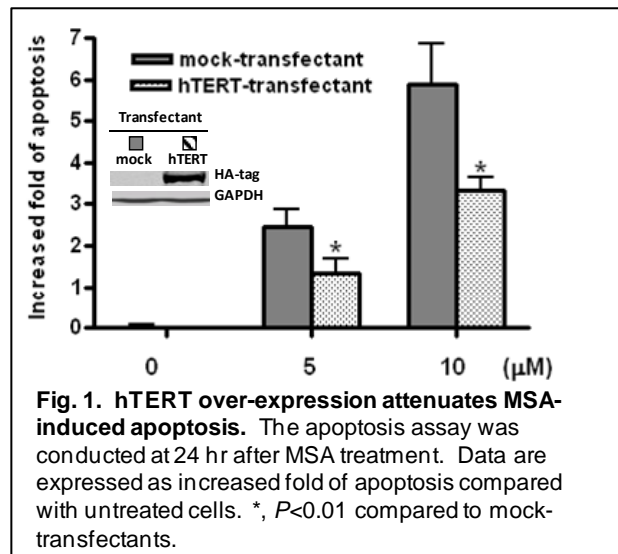
B. BODY:

Aim 1: To assess the cellular mechanism by which hTERT/telomerase down-regulation mediates the anti-cancer effect of methylseleninic acid (MSA)

Experiment 1-1. To investigate the effect of hTERT restoration on MSA-mediated growth inhibition.

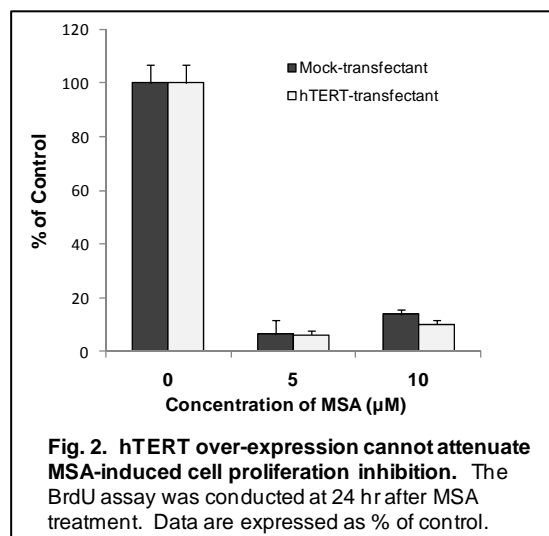
Over-expression of hTERT weakens the apoptosis inducing activity of MSA in LNCaP cells. In order to study the biological significance of hTERT inhibition by MSA, we transiently transfected LNCaP cells with an hTERT expression construct, hTERT/pCI-Neo, and determined the effect of hTERT over-expression on MSA-induced apoptosis by using the Cell Death Detection ELISA^{PLUS} kit (Roche). As shown in Fig. 1, the effect of MSA on apoptosis induction is attenuated by hTERT over-expression.

Over-expression of hTERT does not affect MSA-mediated cell proliferation in LNCaP cells. We also investigated the effect of hTERT over-expression on cell proliferation and colonogenic ability by using the BrdU Cell Proliferation ELISA kit (Roche) and soft agar assay, respectively. As shown in Fig. 2, there is no difference in cell proliferation between mock-transfectant and hTERT-transfectant with MSA treatment, indicating that



restoration of hTERT does not attenuate MSA effect on cell proliferation. Since the BrdU ELISA is much more sensitive than the flow cytometry-based assay and we did not observe an attenuation of MSA-mediated proliferation inhibition by over-expressing hTERT, it became unnecessary to perform the cell cycle analysis by flow cytometry. Our preliminary result of soft agar assay shows no change of MSA-mediated suppression of colonogenic ability with hTERT restoration (data not shown). We plan to repeat the experiment to further confirm our observation. The above data suggest that the mechanism by which hTERT/telomerase repression mediates MSA action is mainly through apoptosis induction.

In the application, we proposed to establish a stable transfectant with inducible hTERT expression to investigate the effect of hTERT restoration on MSA-mediated growth inhibition. However, we found that transient transfection is sufficient to achieve a high expression of hTERT and to attenuate the effect of MSA on apoptosis induction. Therefore, it became unnecessary to generate the stable transfectant for this study.



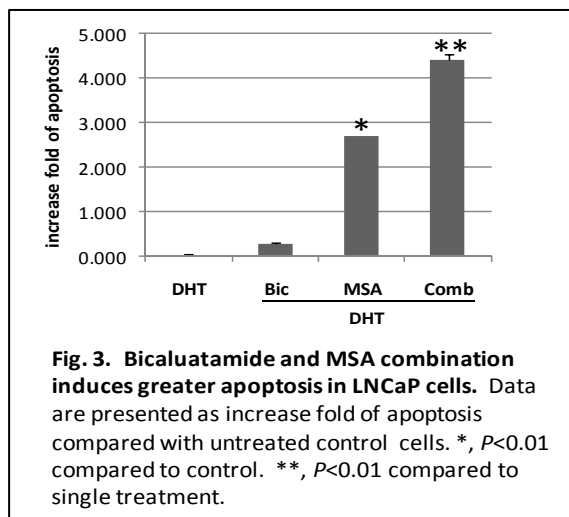
Experiment 1-2. Combined bicalutamide and MSA treatment has enhanced induction of apoptosis and inhibition of hTERT expression.

We added a new dimension to the study by investigating the combinatorial effect of anti-androgen and MSA on cell growth, AR signaling and hTERT/telomerase. The purpose of this study is to determine the potential of using MSA to increase the cancer-killing efficacy of anti-androgen in both androgen-dependent and castration-resistant prostate cancer (CRPC) cells.

Androgen deprivation therapy (ADT) is the mainstay treatment for non-organ-confined prostate cancer or prostate cancer that recurs after initial surgery or radiation therapy (15). ADT targets the action of AR by reducing the level of circulating androgens through surgical or chemical castration and/or by the administration of anti-androgen, such as bicalutamide, flutamide or nilutamide, to inhibit the binding of androgens to AR (16). While a significant initial response to ADT is common, prolonged use of ADT frequently leads to the development of CRPC, which is considered incurable and lethal (17;18). AR expression and signaling are generally maintained in CRPC. Xenograft studies have shown that knocking down AR expression by shRNA could delay the progression of prostate cancer to CRPC and suppress the growth of prostate tumor that has already progressed to the castration-resistant state (19;20). Therefore, rationally designed therapies aimed at diminishing the availability of AR would be helpful not only in enhancing the efficacy of ADT, but also in inhibiting the development of CRPC. MSA is an agent that could effectively reduce AR abundance. Therefore, it is reasonable to believe that anti-androgen and MSA in combination would produce a more pronounced effect on AR-signaling inhibition and thereby hTERT/telomerase suppression, thus triggering cell apoptosis.

Based on our previous dose screening data, we chose 5 μM bicalutamide (Bic) and 2.5 μM MSA for our combination study. As shown in Fig. 3, the combination treatment has greater induction of apoptosis (~4.5-fold increase) than bicalutamide (~0.3-fold) or MSA (~2.7-fold) single treatment.

We then looked at the effect of bicalutamide and MSA combination on suppression of AR signaling. The luciferase assay shows that combination treatment can totally block DHT-

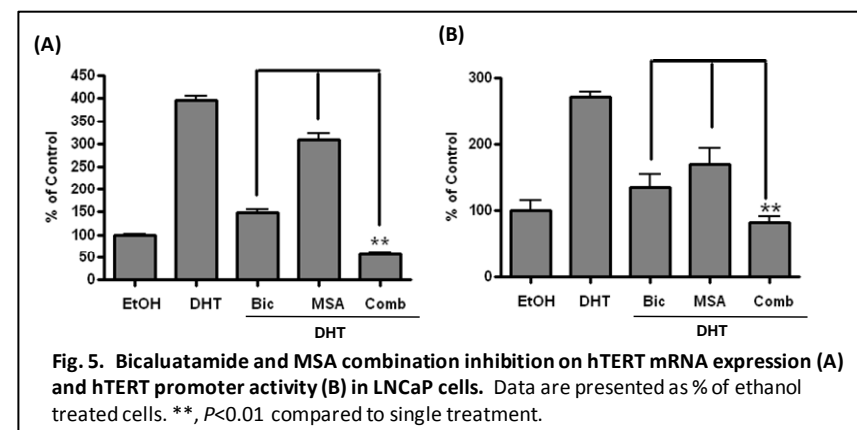
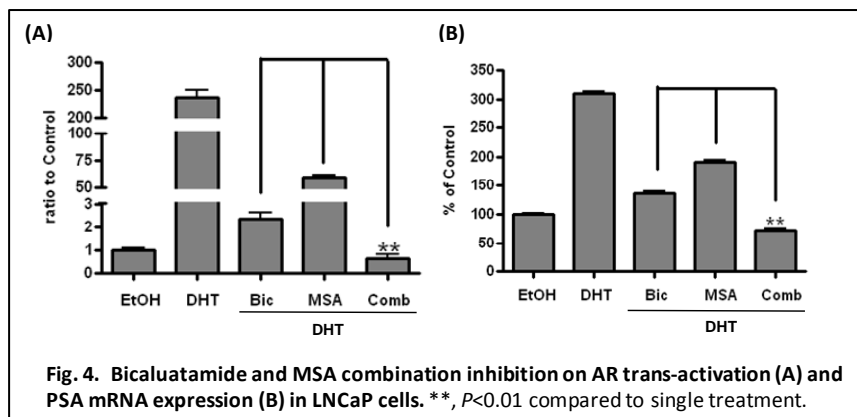


induced AR transcriptional activity compared with single agents (Fig. 4A). This is also confirmed by our quantitative reverse transcription-PCR (qRT-PCR) data of prostate-specific antigen (PSA) mRNA expression. As shown in Fig. 4B, PSA expression is induced to 3-fold by DHT treatment. Bicalutamide and MSA can bring it down to ~1.4- or ~1.9-fold of vehicle control. The inhibition becomes more significant when these two agents are combined, which is only 70% of vehicle. The above data support our hypothesis that bicalutamide and MSA combination has better effect on AR signaling inhibition than single agents.

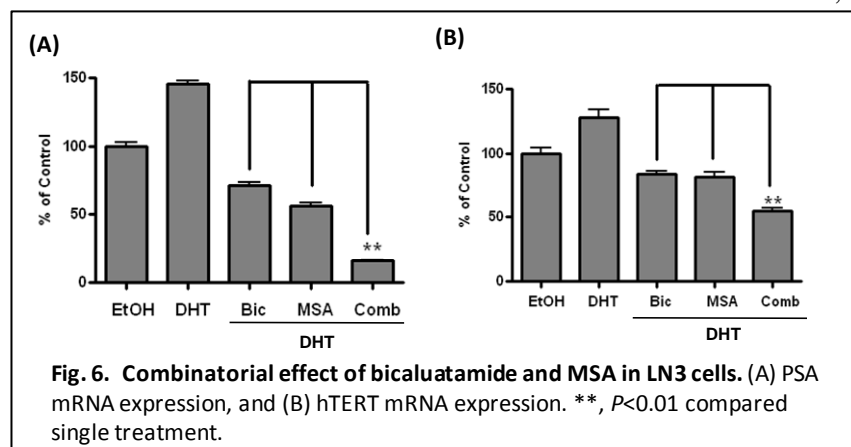
Next we proceeded to evaluate the effect of

bicalutamide in combination with MSA on hTERT expression. Fig. 5A shows that hTERT mRNA expression is induced to 4-fold with DHT treatment. Compared to bicalutamide and MSA treatment alone, combination treatment has the greatest suppression of hTERT expression (~58% of vehicle control). The hTERT promoter luciferase assay result shown in Fig. 5B is consistent with the mRNA data.

We also expanded our study to LN3 cells, which is a castration-resistant but androgen-responsive derivative of LNCaP cells. Consistent with the castration-resistant characteristics of LN3 cells,



only a marginal induction of PSA and hTERT mRNA by DHT was observed (Fig. 6). Treatment with bicalutamide or MSA alone inhibited DHT-induced PSA and hTERT expression; the inhibitory effect was much more striking when the two drugs were used in combination. The above data thus demonstrate a robust



suppression of AR trans-activation by bicalutamide and MSA combination in both androgen-dependent and castration-resistant prostate cancer cells.

In order to delineate the functional significance of hTERT downregulation in mediating the effect of bicalutamide and MSA, we transiently transfected LNCaP cells with an hTERT/pCI-Neo expression construct and assessed the response of the hTERT-

overexpressing cells to the induction of apoptosis. As shown in Fig. 7, the restoration of hTERT not only weakened the apoptosis-inducing ability of bicalutamide or MSA alone, but also that of the combination, thus confirming the critical involvement of hTERT downregulation in mediating the combination effect.

ADT is the mainstay treatment for advanced prostate cancer. It targets the action of androgen receptor (AR) by reducing androgen level and/or by the administration of anti-androgen that competes with androgens for binding to AR. Albeit effective in extending survival, ADT is associated with dose-limiting toxicity and the development of CRPC after prolonged use. Since CRPC is generally lethal and incurable, developing effective strategies to enhance the efficacy of ADT and circumvent resistance becomes an urgent task. Continuous AR signaling constitutes one major mechanism underlying the development of CRPC. Our finding showed that MSA, an agent that effectively reduces AR abundance, could enhance the cancer-killing efficacy of the anti-androgen bicalutamide in both androgen-dependent and castration-resistant prostate cancer cells, thus indicate that MSA in combination with anti-androgen could represent a viable approach to improve the therapeutic outcome of ADT.

In addition the identification of hTERT/telomerase as an important AR target mediating the bicalutamide/MSA effect has great clinical implications. Telomerase activation has been reported in >90% of prostate cancer samples, but not in normal or benign prostatic hyperplasia tissues (21;22). Telomerase activation has been well documented to play an essential role in cell survival and oncogenesis, and inhibition of telomerase has been shown to suppress growth and tumorigenic potential of prostate cancer cells (23-25). Blocking telomerase activation by anti-androgen and MSA through suppressing AR signaling could thus represent an effective and selective treatment modality to target prostate cancer cells. Additionally, hTERT/telomerase could be measured in blood and urine (26;27), and therefore could serve as a non-invasive, tumor-specific, functionally relevant molecular biomarker for monitoring the efficacy of the intervention.

Experiment 2. To investigate the mechanism by which hTERT/telomerase suppression mediates the anti-cancer effect of MSA.

Our previous studies showed that 10 μ M MSA induces a marked growth inhibition of prostate cancer cells at 48 hr. Telomerase suppression by MSA is unlikely to result in appreciable telomere shortening within such a

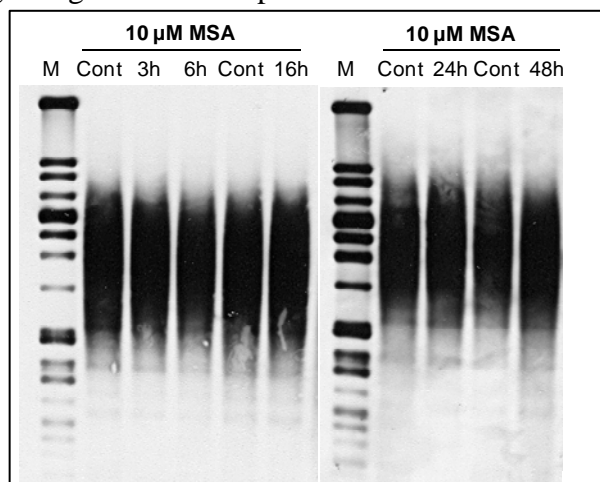
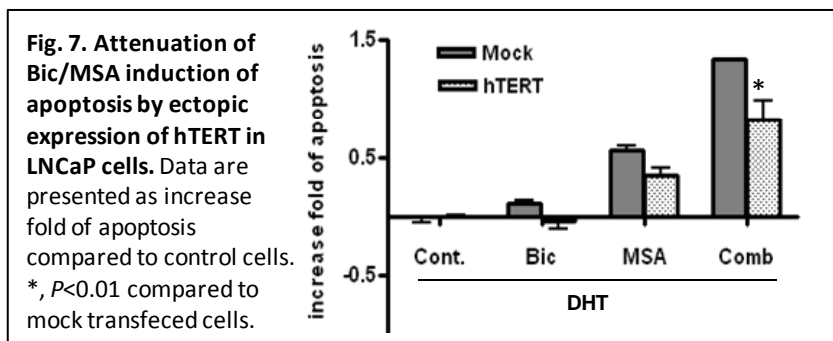
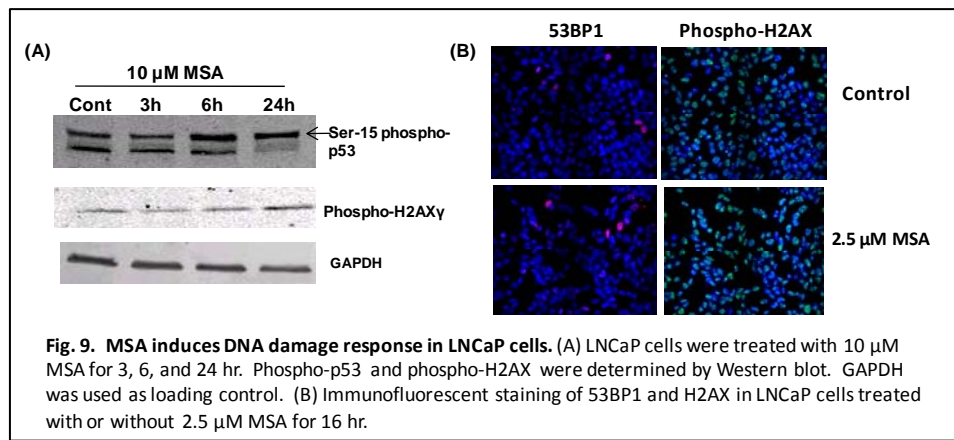


Fig. 8. MSA does not affect telomere length in LNCaP cells. LNCaP cells were treated with 10 μ M MSA for 3, 6, 16, 24 and 48 hr. Telomere length was detected by using TeloTAGGG Telomere Length Assay (Roche).

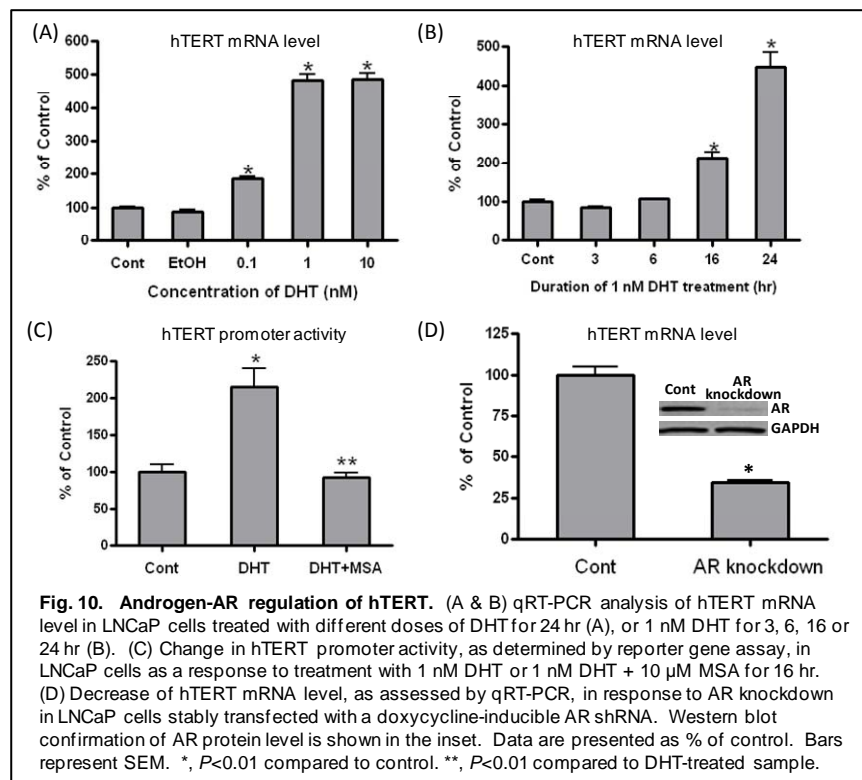
short period of time. As expected we did not observed any change of telomere length up to 72-hour MSA treatment (Fig.8). Therefore, a mechanism independent of telomere shortening, such as telomere capping status, should be considered.

Uncapped telomeres have been reported to trigger a rapid DNA damage response and lead to cell cycle arrest and/or apoptosis (28). We then studied the effect of MSA on expression of DNA damage response markers. As shown in Fig. 9A, 10 μ M MSA induced phosphorylation of p53 and H2AX γ , indicating that MSA is able to induce DNA damage response in LNCaP cells. We then performed immunofluorescent staining of phosphor-H2AX γ and 53BP1 in LNCaP cells with or without MSA treatment. Since 5 μ M and 10 μ M MSA caused cell floating after 16-hr treatment, we could only get result from 2.5 μ M MSA-treated cells (Fig. 9B). To improve this assay, we plan to do short-term treatment (3 hr or 6 hr) with 10 μ M MSA. In addition, we are in the process of co-staining the cells with telomere-foci and DNA-damage markers to delineate whether MSA induces telomere uncapping which leads to the DNA-damage response.



Aim 2: To study the mechanism by which AR signaling suppression contributes to the down-regulation of hTERT by MSA

Androgen signaling up-regulation of hTERT expression in LNCaP cells. To study the effect of AR signaling on hTERT expression, qRT-PCR and luciferase assay were performed in LNCaP cells. As shown in Fig. 10A&B, DHT can induce hTERT mRNA expression dose- and time- dependently. At 24 hr treatment, 0.1 nM DHT could induce hTERT mRNA to ~2-fold of control. With the dose increased to 1 nM, the induction became more dramatic (~5-fold). The magnitude remains at the same level with 10 nM DHT treatment. The



obvious induction occurs at 16 hr and becomes more dramatic at 24 hr. The luciferase assay result shows that the 4-kb hTERT promoter region is up-regulated by 1 nM DHT at 16 hr (Fig. 10C, the first two columns). The induction was almost completely blocked by 10 μ M MSA. We then studied the consequence of AR knockdown on hTERT expression. As shown in Fig. 10D, the knockdown of AR by treating LNCaP cells stably transfected with a doxycycline-inducible AR-shRNA lentiviral system with doxycycline leads to a significant reduction of hTERT mRNA. The data therefore suggest that suppression of AR signaling by either MSA treatment or AR knockdown could efficiently inhibit hTERT expression.

AR over-expression cannot reverse MSA inhibition of hTERT in LNCaP cells.

Then we over-expressed AR to assess whether this could reverse the action of MSA on hTERT expression. However, there is no change of MSA repression of hTERT expression with AR restoration (Fig. 11). The data therefore indicate that other transcription factor(s) might be involved in this process. We are in the process of using chromatin immunoprecipitation (ChIP) assay to investigate the

association of AR and hTERT promoter region that was reported to associate with AR. The purpose of this study is to demonstrate that suppressed hTERT promoter activity is attributable by reduced AR occupancy of the hTERT promoter as a consequence of MSA downregulation of AR.

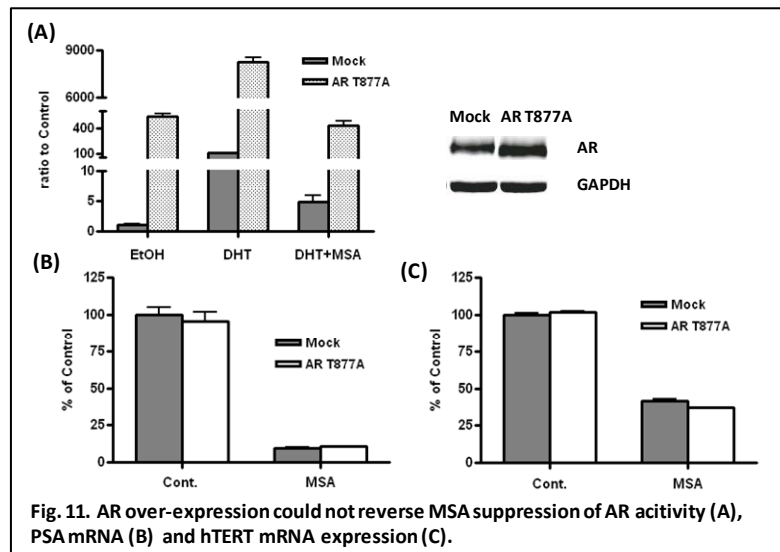


Fig. 11. AR over-expression could not reverse MSA suppression of AR activity (A), PSA mRNA (B) and hTERT mRNA expression (C).

Androgen signaling represses hTERT expression in LAPC-4 cells. In addition to LNCaP cells, we also investigated androgen effect on hTERT expression in another androgen-dependent cell line, LAPC-4. Interestingly, we found that in contrast to up-regulation of hTERT expression in LNCaP cells, DHT actually inhibits hTERT mRNA expression in LAPC-4 cells (Fig. 12). **Why do LNCaP and LAPC-4 cells respond differentially to DHT treatment?**

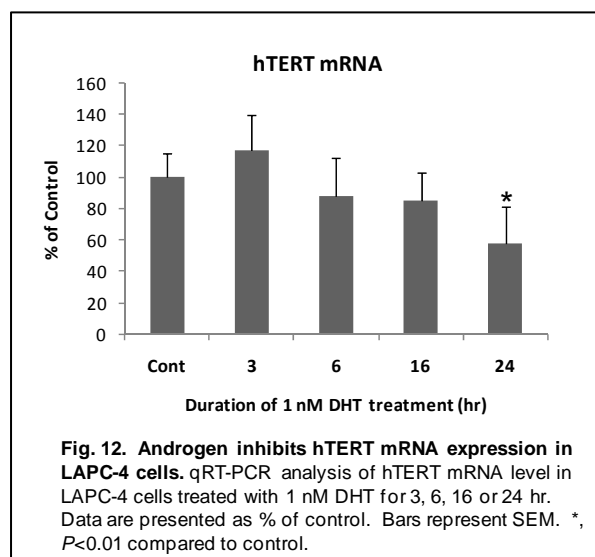


Fig. 12. Androgen inhibits hTERT mRNA expression in LAPC-4 cells. qRT-PCR analysis of hTERT mRNA level in LAPC-4 cells treated with 1 nM DHT for 3, 6, 16 or 24 hr. Data are presented as % of control. Bars represent SEM. *, $P < 0.01$ compared to control.

LNCaP cells express a mutant but functional androgen receptor, whereas LAPC-4 cells express a wild-type AR. In fact, Moehren *et al* reported that wild-type but not mutant androgen receptor inhibits the expression of hTERT (29), which is consistent with our finding. However, the majority of prostate cancer cells express wild-type but no mutant AR. It is also reported that androgen ablation therapy significantly inhibits hTERT activity in prostate carcinomas. It seems that in tumor environment, androgen stimulates hTERT/telomerase, while ADT inhibits it. Hypoxia is a common condition in tumor tissues, especially in prostate cancer. It is possible that in hypoxia condition androgen has

different effect on hTERT expression in cells expressing wild-type AR. To address this question, we plan to investigate the effect of DHT on hTERT expression in both normoxia and hypoxia conditions. The findings would not only allow us to further understand the regulation mechanism of hTERT/telomerase by androgen; but also provide a mechanistic basis for developing targeted therapy for prostate cancer.

C. KEY RESEARCH AND TRAINING ACCOMPLISHMENTS:

- Over-expression of hTERT attenuates the apoptosis induction activity of selenium in LNCaP cells.
- Bicalutamide and selenium combination has greater apoptosis induction in LNCaP cells than single agents.
- Bicalutamide and selenium combination significantly blocks AR signaling, thus leading to inhibition of hTERT expression.
- hTERT/telomerase is an important AR target mediating the bicalutamide/MSA effect.
- AR signaling regulates hTERT expression at transcriptional level.
- In addition to AR protein level, other mechanism(s) might participate in selenium repression of hTERT.
- Wild-type and mutant AR have different effect on hTERT mRNA expression.

D. REPORTABLE OUTCOMES:

- ***Presentations:***
20th Annual Tulane Health Sciences Research Day, 2008, poster presentation, “*Down-regulation of telomerase by selenium in prostate cancer cells*”.
- The Breast Ovarian Cancer Journal Club/Seminar Series, 2008, oral presentation, “*Down-regulation of telomerase by selenium in prostate cancer cells*”.

E. CONCLUSIONS:

The result from our current study suggests that hTERT, a novel target of AR signaling, plays an important role in mediating selenium action in human prostate cancer cells. Our data also provided an array of evidence supporting selenium in combination with an anti-androgen as a potential new modality for not only the prevention but also the treatment of prostate cancer. More importantly, we indentified hTERT/telomerase as an important AR target mediating the bicalutamide/MSA effect. Our current finding brings a new direction to this project and it has great clinical implications. Further continuation of this study will provide a justification for a mechanism-driven strategy in using selenium, in combination with anti-androgens, to control prostate cancer development and progression.

We also found an interesting phenomenon that wild-type and mutant AR have different function in term of regulation of hTERT expression. Since the mechanism of hTERT regulation by AR is not well studied, it would be more important to address this issue. Based on the above information, we would like to request a change on our future work. We would like to continue our study of the mechanism by which AR up-regulates hTERT expression by studying the

association of AR with androgen-responsive element (ARE) in hTERT promoter region, but shift our future research focus to investigate why wild-type and mutant AR respond differently to androgen treatment regarding hTERT expression. We would like to study the androgen effect on hTERT expression under both normoxia and hypoxia conditions and to elucidate whether the effect is mediated by AR. ADT has been reported to cause hypoxia in tumor tissues (30), and hypoxia is known to promote androgen-independent growth, chemo-resistance and metastasis (31). Therefore it is imperative to seek for approaches targeting tumor hypoxia. We would like to study the effect of selenium on hTERT/telomerase under hypoxia condition. If selenium is able to suppress hTERT/telomerase under hypoxia condition, it would have great implication for enhancing the efficacy of ADT in prostate cancer. We would greatly appreciate your kind consideration of our request.

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G. APPENDICES

Down-regulation of telomerase by selenium in prostate cancer cells

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The Nutritional Prevention of Cancer Trial showed that selenium supplementation reduced prostate cancer incidence by ~50%, although the underlying mechanism remains unclear. Telomerase activation is a rate-limiting step in cellular immortalization and oncogenesis. In the present study, we demonstrated a dose- and time-dependent drop in telomerase activity as a result of selenium treatment in human prostate cancer cells. The reduction was mostly attributable to a significant decrease in the level of the catalytic subunit, human telomerase reverse transcriptase (hTERT), the major determinant of telomerase enzymatic activity. Such effect of selenium was detected in 4/4 of the androgen-dependent and -independent human prostate cancer cell lines examined, suggesting the universality of the phenomenon. Results from mRNA stability analysis and nuclear run-on assay indicated hTERT downregulation occurred mainly at the transcriptional level. The expression of the hTERT gene is known to be regulated by a number of transcription factors, including androgen receptor (AR). AR plays an important role in the development and progression of prostate cancer, and selenium treatment led to a marked decrease in the expression and *trans*-activating/DNA-binding activity of AR. We therefore investigated the potential involvement of AR in selenium downregulation of hTERT. We found that activation of AR signaling by dihydrotestosterone (DHT) increased hTERT mRNA transcription. This effect was both dose- and time-dependent. On the other hand, AR knockdown decreased hTERT mRNA expression and enhanced the suppression effect of selenium on hTERT. However, restoration of AR by overexpression was not able to reverse selenium effect on hTERT, suggesting the involvement of mechanisms in addition to decreasing AR abundance, such as disrupting the interaction between AR and AR co-regulators. We are currently using ChIP assay to study the recruitment of AR to the hTERT promoter under selenium treatment. Telomerase activation has been detected in the vast majority of tumor samples, and is one of the most widespread tumor markers. In prostate cancer, the activation is already evident at early stages of the disease. Consequently, telomerase represents an attractive target for prostate cancer prevention and treatment. Our novel findings therefore provided justification for a mechanism-driven strategy in using selenium to control prostate cancer development and progression. In addition, considering the fact that telomerase/hTERT could be measured in the circulation, our data could lead to the identification of a new non-invasive molecular biomarker for future selenium intervention trials to gauge the efficacy of intervention. (Supported by NCI grant CA114252 and ACSRSG-07-218-01-TBE).